

Effect of a metabolically created systemic acidosis on calcium homeostasis and the diurnal variation in urine pH in the non-lactating pregnant dairy cow

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Reducing the dietary cation-anion difference (DCAD) has been shown to be an effective means of preventing parturient paresis in confinement systems where cows are offered a total mixed ration containing DCAD-reducing mineral compounds (anionic salts). Such a supplementation strategy is not possible in cows being group fed forages precalving, and little is known about the effect of supplementing these cows with large amounts of anionic salts twice daily.

Eight non-lactating, pregnant Holstein-Friesian cows were allocated to two levels of DCAD (–20 and +18 meq/100 g DM) for 24 d, with an intensive Ca balance undertaken in metabolism stalls following a 2-week acclimatization to diet. The basal diet was 3 kg DM of crushed barley and 7 kg DM of pasture-hay. Urine and faeces were collected separately, weighed daily for 5 d and analysed for Ca content. Urinary Ca, creatinine and hydroxyproline concentration and plasma Ca concentration were determined during the period of the balance study. The diurnal pattern in urine and rumen pH was determined over 2 d. Decreasing DCAD reduced ($P<0.001$) the pH of urine, and increased ($P<0.05$) Ca absorption. Plasma Ca concentration was not affected by DCAD, and DCAD did not affect the output of urinary hydroxyproline, a marker of bone resorption. Twice-daily supplementation of anionic salts was sufficient to reduce the pH of blood and increase gastrointestinal Ca absorption. There was no diurnal variation in the pH of urine, suggesting that time of sampling to determine efficacy of DCAD in reducing systemic pH was not important.

Keywords: Dietary cation-anion difference, anionic salts, pH, calcium, non-lactating dairy cows, DCAD.

In the transition from pregnancy to lactation, clearance of Ca to the placenta ceases but the lactational Ca demand increases rapidly (Ramberg et al. 1984). Hypocalcaemia develops in most cows owing to this demand and, if blood Ca becomes insufficient to support nerve and muscle function, parturient paresis (milk fever; MF) results (Schultz et al. 1988; Goff & Horst, 1997).

Following analysis of 30 years' data, Roche & Berry (2006) concluded that MF incidence in pasture systems approximated 2–3%, but varied greatly from year to year and was influenced by climate and cow factors such as condition score, parity and breed. The disorder was first reported in Germany in 1793 (Schultz et al. 1988) and has since been a well-researched subject because of its economic importance. Block (1984) reported a 14% and 7%

reduction in milk yield in cows that experienced clinical and subclinical parturient hypocalcaemia, respectively, and Belonje & Van der Walt (1971) reported poorer fertility (prolonged inter-calving period) in cows that contracted MF. In addition, Schultz et al. (1988) reported a mortality rate of 3.5–5% in clinically affected cows, and observed that productive life of affected cows was reduced by approximately 3.5 years. Similarly, Cox (1981) reported that 4–28% of cases of MF relapse and may become downer cows. Of those affected, 20–67% die subsequently or must be slaughtered.

MF control strategies vary (see reviews by Horst et al. 1997; Roche, 2003) and include the feeding of a precalving diet containing a low Ca concentration (Wiggers et al. 1975; Littledike & Goff, 1987), supplementation with Mg precalving (Lean et al. 2006) and/or supplementation of Ca at calving (Roche et al. 2002, 2003b). These treatments reflect the belief that dietary Ca concentration, either too

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great precalving or insufficient availability postcalving, is the principal determinant of a cow's susceptibility to parturient hypocalcaemia.

Although these nutritional practices have dramatically reduced the incidence of MF (Roche & Berry, 2006), they have failed to eradicate it. More recently, the relative importance of precalving dietary Ca has come under review (Oetzel, 1991; Goff & Horst, 1997; Lean et al. 2006), and it has been proposed that it may not be as important a risk factor as high intakes of K and Na precalving, and their effects on blood acid-base status (Goff & Horst, 1997). Changes in blood pH affect Ca metabolism (Bushinsky et al. 1993). A precalving diet with a relative predominance of S and Cl (anions) to Na and K (cations; *i.e.* a negative dietary cation-anion difference or DCAD) has been shown to reduce blood pH in pasture-based cows (Roche et al. 2003a), and increase the absorption (Schonewille et al. 1994) and urinary excretion of Ca (Vagg & Payne, 1970; Vagnoni & Oetzel, 1998).

An issue peculiar to the management of cows fed forage precalving is the inability to mix the required mineral compounds with the base-feed. Roche et al. (2003a,b) administered mineral compounds via a drench twice daily. Little is known about the efficacy of these compounds when supplemented twice daily, compared with throughout the day as in systems using total mixed rations. Similarly, the effect of twice-daily supplementation on the diurnal variation in the pH of urine and rumen fluid requires investigation. The objective of the work reported here was to examine the effect of a reduced DCAD on Ca absorption, and the amount of Ca and hydroxyproline excreted by the non-lactating pregnant dairy cow.

Materials and Methods

The experiment was conducted at Agriculture Victoria Ellinbank (37° 50' S, 145° 00' E), approximately 110 km east of Melbourne, Australia, in May–June 1999. All procedures in this study were approved by the Ellinbank Animal Ethics Committee and animals were handled according to the Code of Practice for the Care and Use of Animals for Experimental Purposes.

Experimental design, feeding and treatments

Eight multiparous, non-lactating, 220±7 d pregnant, rumen-fistulated Holstein-Friesian cows were randomly allocated to two DCAD treatments (High and Low), ensuring groups were balanced for age (5.8±0.44 years) and liveweight (584±52 kg). The use of non-lactating dairy cows that are not near calving does not allow direct extrapolation of results to parturition, but findings can be interpreted physiologically and are therefore important.

All cows were offered a restricted daily diet of 3 kg DM of crushed barley and 7 kg DM pasture-hay (predominantly *Lolium perenne* L.). This is a diet not untypical

of what is offered to dairy cows in Australia and New Zealand around parturition, when pasture growth rates are insufficient to meet cow demands. The barley was individually fed, in two feeds, at 09.00 and 15.00 and the hay was fed once a day at 09.30. Cows had approximately 12-h access to their hay allocation. Water was available *ad libitum* and water intake was recorded thrice daily.

Cows on the Low DCAD treatment were supplemented with 200 g MgCl₂·6H₂O and 100 g NH₄Cl to reduce the DCAD to -20 meq/100 g DM. DCAD was calculated using the equation:

$$\text{DCAD (meq/100 g DM)} = (\text{Na}/0.023) + (\text{K}/0.039) \\ - (\text{Cl}/0.0355) - (\text{S}/0.016)$$

where dietary mineral concentration is expressed as % DM.

To ensure that treatments did not differ in their dietary Mg concentration (0.5% DM), High DCAD cows were supplemented with 60 g MgO. Cows received their appropriate mixture of mineral compounds twice daily at 09.00 and 15.00 via the rumen fistula.

Measurements

Background measurements of urine pH and Ca and creatinine concentrations were taken on all animals prior to beginning the experiment. Cows were allowed to adapt to their respective treatments for 14 d (acclimatization period; days 1–14), following which the cows were individually fed indoors in metabolism stalls (2 m × 1.3 m) for 9 d (days 15–23). Cows were allowed access to their barley for 15 min at 9.00 and 15.00 before being allowed access to hay until 21.30. Days 15 and 16 were conditioning days for the cows to become accustomed to the stalls. On day 16 the cows were fitted with a harness to facilitate separation of urine and faeces. The intensive Ca balance monitoring period ran from days 17–21. Days 22, 23 and 24 were used for intensive monitoring of the pH of urine and ruminal contents.

During the 4 d prior to the start of the experiments (covariate period; days -3 to 0), the 14-d acclimatization period outdoors, the first 2 d of the metabolism stall period (days 15 and 16) and on days 22–24, cows were manually stimulated to urinate at 9.00, and a sample of urine was collected from midstream in a 50-ml sterile container. Within 30 min of collection, the pH was determined and representative subsamples analysed for Ca and creatinine, respectively.

During the Ca balance period (days 17–21), faeces and urine were collected separately in plastic containers. The weight of faeces and the volume of urine voided during the previous 24 h were recorded at 9.00 daily. Faeces were thoroughly mixed and samples dried at 105 °C and 65 °C for DM and Ca determination, respectively. After mixing, the pH of urine was determined and samples analysed for Ca, hydroxyproline and creatinine.

Table 1. Mean dry matter (DM, % fresh weight), crude protein (CP), dry matter digestibility *in vitro* (DMD), metabolizable energy (ME; MJ/kg DM), dietary cation-anion difference (DCAD; meq/100 g DM) and mineral concentrations (% DM, unless stated otherwise) of the feeds offered to non-lactating dairy cows

Feed	DM	CP	ME	K	Na	Cl	S	Mg	Ca	P	DCAD
Hay	79	8.6	8.5	2.33	0.31	1.30	0.16	0.17	0.40	0.21	26.5
SD	3.1	0.15	0.60	0.12	0.09	0.10	0.02	0.01	0.04	0.03	3.9
Barley	87	11.7	12.2	0.50	0.02	0.16	0.14	0.11	0.05	0.32	2.2
SD	1.0	0.15	0.08	0.06	—	0.03	0.02	0.01	0.01	0.02	0.7
Diet	82.4	9.6	9.7	1.76	0.30	1.07	0.16	0.15	0.34	0.22	18.1
SD	2.73	0.29	0.29	0.10	0.02	0.06	0.01	0.01	0.02	0.01	1.08

One evacuated blood tube containing a sodium heparin pellet (100 i.u. sodium heparin/ml blood), to prevent coagulation, was collected by jugular venipuncture daily from each cow during the Ca balance period. Plasma was extracted by centrifuging at 1120 *g* at 4 °C for 10 min and analysed for Ca content.

Urine, faecal and plasma Ca concentrations were determined against a series of Ca standards by atomic absorption spectrophotometry (Perkin Elmer 372, Rodgau-Jügesheim, Germany) at 422.7 nm. Urine hydroxyproline was determined on a microplate reader (Biorad 550, Hercules CA, USA; Parekh & Jung, 1970) and urinary creatinine was measured using an autoanalyser (Boehringer Mannheim Hitachi 911, Mannheim 31, Germany) as described by Bartels et al. (1972).

Daily intake was recorded for each cow and representative samples (approximately 200 g) of each feed offered and refused were dried at 105 °C for 24 h to determine DM content and calculate DM intake (DMI) by difference. Daily samples of all feeds offered were bulked weekly during the 14-d acclimatization and over the 9 d of the indoor intensive measurement period, dried at 65 °C for 72 h, ground to pass through a 0.5-mm sieve, and analysed for macrominerals, DM digestibility *in vitro* (DMD) and nitrogen. Macrominerals were determined using x-ray spectroscopy (Hutton & Norrish, 1977; Norrish & Hutton, 1977). DMD was determined by the method of Clarke et al. (1982) and N content was determined by Kjeldahl using an automated Tecator instrument, (Foss, Denmark; Association of Official Analytical Chemists, 1990). Metabolizable energy (ME) was calculated from DMD (ME=DMD × 0.17; Standing Committee on Agriculture, 1990) and crude protein was calculated from N (Crude protein=N × 6.25). Nutritive characteristics and mineral concentrations of the feeds offered are presented in Table 1.

Intensive rumen and urine sampling

Urine and rumen samples were taken at 8.00, 12.00, 16.00, 20.00 and 24.00 day 22, 4.00, 6.00, 10.00, 14.00, 18.00 and 22.00 on day 23 and 2.00 and 6.00 on day 24. Urine samples were obtained by perineal stimulation for up to 10 min. Samples could not be obtained on six occasions. A representative sample (50 ml) of ruminal fluid

Table 2. Effect of dietary cation-anion difference (DCAD) on the daily dry matter intake (DMI) (kg DM/cow), water imbibed daily (l/cow), daily outputs of faeces (kg DM/cow) and urine (l/cow), daily faecal water output (kg/cow) and dry matter digestibility *in vivo* (IVDMD; %) of non-lactating pregnant dairy cows

	High DCAD	Low DCAD	SED	P value
DMI	9.3	9.2	0.54	0.82
Water imbibed	36.3	39.9	2.16	0.13
Faecal output	3.2	3.5	0.42	0.49
Faecal water	23.2	25.3	1.53	0.21
Urine output	15.0	13.3	2.74	0.55
IVDMD	61.0	60.4	4.17	0.87

was obtained from three sites in the rumen (anterior, central and posterior) approximately equidistant between the dorsal and ventral surfaces, through a 500-mm copper pipe (10-mm diameter with 5-mm holes in the last 80 mm) attached to a 50-ml syringe. The pH of urine and ruminal fluid was measured within 30 min of collection using a Metrohm 827 pH meter (Metrohm, Herisau, Switzerland).

Calculations and statistical analysis

Urinary Ca concentration was expressed as a ratio to creatinine concentration, providing a corrected urinary Ca concentration (CUCa) to overcome variations in urine volume between animals (Roche et al. 2003a).

All data were analysed using Analysis of Variance (Genstat V, 1997) with cows as a random effect and DCAD as a fixed effect. Repeated measurements through time were modelled using spline models within the linear mixed model framework. There was an interaction with time and so individual time points are presented for ease of interpretation.

Results and Discussion

DMI, water imbibed and urine and faecal output were not affected by DCAD treatment (Table 2).

Treatments were implemented successfully, with DCAD of −20 and +18 meq/100 g DM consumed on the low and high DCAD treatments, respectively. Further proof was the decline ($P<0.001$) in urine pH on the low DCAD

Table 3. Effect of dietary cation-anion difference (DCAD) on the urine pH, net Ca balance (g Ca/cow per d, unless stated otherwise) and urinary hydroxyproline output of non-lactating pregnant dairy cows

	High DCAD	Low DCAD	SED	P value
Urine pH	8.5	5.7	0.05	<0.001
Ca intake	28	28	1.48	0.64
Urine Ca	0.8	5.3	0.69	0.001
Faecal Ca	46	37	3.3	0.04
Total Ca excreted	47	43	3.44	0.26
Ca retention	-19	-14	3.23	0.17
Plasma Ca, mmol/l	2.50	2.45	0.058	0.31
Urinary hydroxyproline, µg/cow per d	8.7	7.4	1.13	0.29

treatment the day following treatment initiation. There was no difference in the intake of Ca between treatments. However, faecal Ca output was decreased ($P<0.05$) and urinary Ca output was increased ($P<0.001$) on the lower DCAD diet, suggesting that gastrointestinal absorption of Ca was improved through the daily provision of anionic salts in two equal doses. The increased absorption did not affect plasma Ca (Table 3).

Schonewille et al. (1994) also found an increased absorption of Ca when anionic salts were fed, but reported that only 60% of increased urinary Ca output could be accounted for by increased absorption. As a result many researchers have postulated that bone resorption may be contributing the Ca unaccounted for by absorption into the excreted Ca pool. In support of this premise, Fredeen et al. (1988) showed that rates of Ca absorption and resorption were elevated in goats made acidotic. Schonewille et al. (1994), however, reported no effect of DCAD on markers of bone resorption. In the present experiment the reduction in faecal Ca output was greater than the increase in Ca excreted in urine, and the urinary output of hydroxyproline (an accepted marker of bone resorption; Russell, 1997) was not affected by treatment ($P=0.29$). These results suggest a lack of effect of DCAD on bone resorption, and support our earlier findings (Roche et al. 2003a,b) in dairy cows and the results presented by Chu et al. (1975) and Kim & Linkswiler (1979) in man. The reason for the inconsistency in the reported effect of DCAD on bone resorption is unclear. Factors, such as the stage of gestation or milk yield may contribute to the variable results reported. One reason in particular may be that urinary hydroxyproline represents only 10% of hydroxyproline released (Liesegang, 2003) and can result from the degradation of tissues other than bone. It is therefore not specific to bone and study results must be considered individually and with caution, particularly in the periparturient cow when tissue catabolism may be contributing significant amounts of hydroxyproline to the pool being excreted. However, because cows in the study reported here were non-lactating, in good health, and at least 2 months precalving, and because at least half of body collagen

resides in bone, where its turnover is faster than in soft tissues (Garnero & Delmas, 1996), we are confident that use of hydroxyproline concentration in urine is an appropriate index of bone resorption. Further research is required to understand the extenuating factors affecting bone resorption and accretion under acidotic conditions.

Urine pH over the experimental period averaged (\pm SD) 8.4 (± 0.22) and 6.4 (± 0.81) on high and low DCAD treatments, respectively, and were consistent with the mean urine pH predicted from the meta-analysis of Spanghero et al. (2004). However the difference in urine pH varied with time. Figure 1 depicts the change in urine pH and CUCa during the experimental period. The pH of urine declined for 2 d following initiation of the low DCAD treatment, reached a plateau, and on day 6 increased from 5.98 to 7.83. Urine pH over this period averaged 8.2 (± 0.25) and 7.1 (± 0.82) on high and low DCAD treatments, respectively. This rise in urine pH is consistent with a similar trend reported by Goff & Horst (1998). They reported an initial decline in urine pH following supplementation of cows with anionic salts, and a subsequent increase in urine pH, which they attributed to buffering of cations released from bone under acidotic conditions. On day 9 of the current study, the pH began to decline again to a trough of approximately 5.5 at day 19 (average \pm SD urine pH after day 9 was 8.5 \pm 0.12 and 6.0 \pm 0.56 on high and low DCAD treatments, respectively). Data reported by Goff & Horst (1998) did not extend beyond day 9 and so it is not possible to say whether their data would have followed a similar trend to those reported here.

Figure 1 shows a concomitant rise in CUCa with declining pH. However, as for pH, the effect of DCAD on CUCa was inconsistent during the first 8 d of treatment, and the association between urine pH and CUCa was weak ($R^2=0.04$) and non-significant ($P=0.58$). However, after day 9 there was a strong ($R^2=0.81$; $\text{CUCa} = -0.47 \times \text{Urine pH} + 3.65$; $P<0.001$) negative relationship between urine pH and CUCa. These results suggest that there is a minimum period (approximately 9 d) of low DCAD required to ensure that Ca absorption has increased and that cows are at a reduced risk of milk fever. This is consistent with the output of a recent meta analysis (Lean et al. 2006) that showed a significant effect of exposure to a transition ration containing anionic salts on the reduction in the odds of milk fever occurring.

Figure 2 shows the effect of a high or low DCAD on the pH of urine and rumen fluid throughout the day. The lack of effect of DCAD on the pH of ruminal contents is in contrast to the findings of Tucker et al. (1988a) who reported a linear decline in the pH of ruminal fluid with decreasing DCAD from +20 to -20 meq/100 g DM. In the work reported here it appears that, although very little variation occurred, the minimum urine pH (the maximum response to a decreasing DCAD) occurred approximately 3–4 h after feeding anionic salts, supporting the earlier work of Tucker et al. (1988b). However, the variation was small.

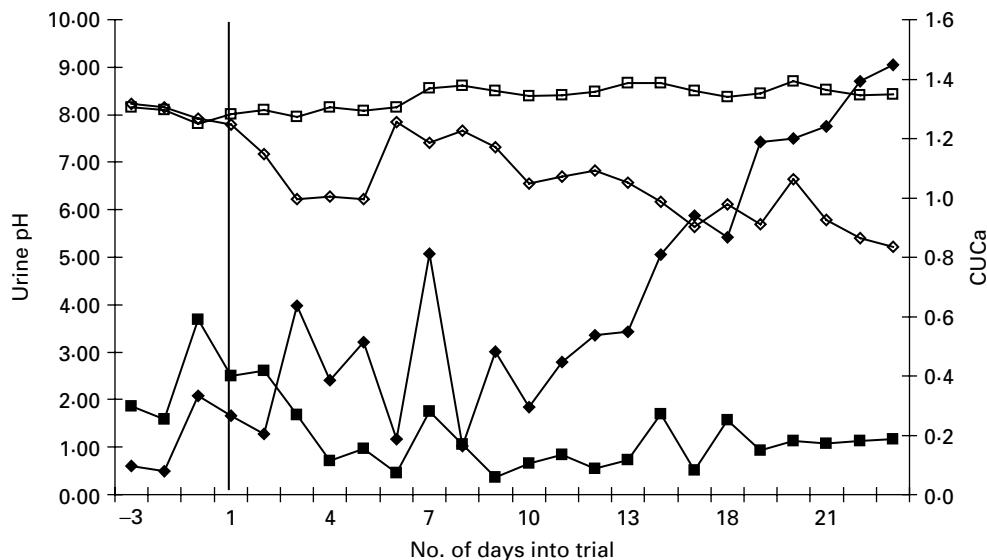


Fig. 1. Effect of a high or low dietary cation-anion difference (DCAD) on the pH of urine (\square and \diamond =high and low DCAD, respectively) and the corrected urinary Ca concentration (CUCa, the ratio of ratio of urine Ca concentration to urine creatinine concentration; \blacksquare and \blacklozenge =high and low DCAD, respectively) of non-lactating pregnant dairy cows. Treatments commenced on Day 1.

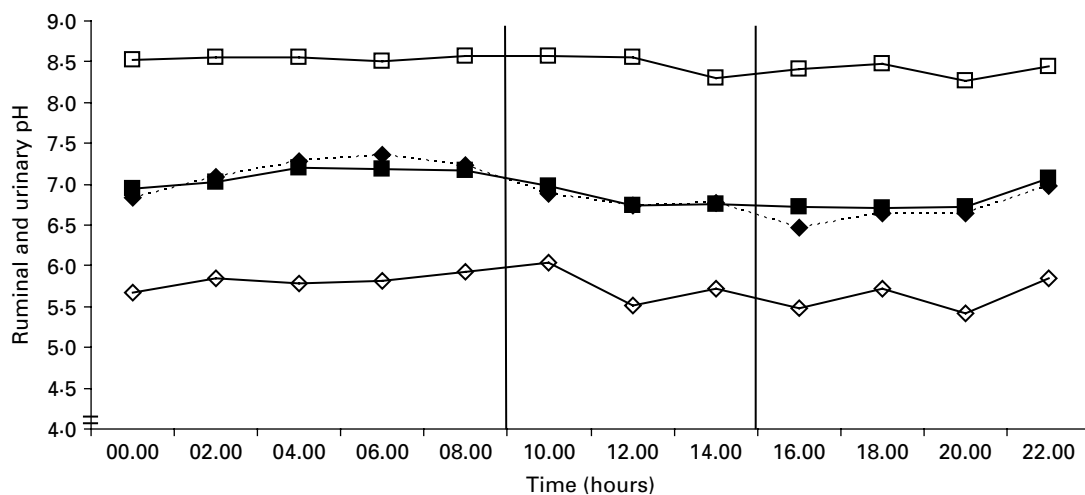


Fig. 2. The effect of a high or low dietary cation-anion difference (DCAD) on the pH of urine (\square and \diamond =high and low DCAD, respectively) and ruminal fluid (\blacksquare and \blacklozenge =high and low DCAD, respectively) of non-lactating pregnant dairy cows over a 24-h period. Ca salts were administered at 9.00 and 15.00.

When DCAD was maintained at -20 meq/100 g DM, the pH of urine remained below 6.2 throughout the 24 h, supporting the review of Horst et al. (1997) and the findings of Roche et al. (2003a), which claimed that urine pH must be maintained between 5.5 and 6.2 for the pH of blood to be sufficiently lowered to increase Ca absorption. The lack of diurnal variation in urine pH from cows on the low DCAD treatment is also an important finding, highlighting that the desired reduction in systemic pH can be achieved in forage-based systems (indoors and grazing) through twice daily supplementation with anionic salts. It also suggests that if a reduction in DCAD is being used to reduce periparturient hypocalcaemia, urine pH can be

measured at any time of the day to ascertain whether DCAD has been reduced sufficiently to alter blood pH.

Conclusions

A low DCAD (-20 meq/100 g DM) caused a metabolic systemic acidosis and an increase in Ca absorption, but did not appear to affect bone resorption. The pH of ruminal contents was unaffected by the reduced DCAD. The pH of urine was reduced by the low DCAD and remained relatively constant throughout the day. This suggests that if anionic salts are being offered twice daily, the pH of urine

can be tested at any time of day to determine the efficacy of the daily ration in reducing the pH of blood. It also suggests that a urinary pH below 6.2 is a good indicator that DCAD has been reduced to the recommended levels of -15 to -20 meq/100 g DM.

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